J. Clin. Chem. Clin. Biochem.
Vol. 22, 1984, pp. 349–356

An Evaluation of Four Different Luminescence Immunoassay Systems: CELIA (chemiluminescent immunoassay), SPALT (solid-phase antigen luminescence technique), ILMA (immunoluminometric assay) and ILSA (immunoluminometric labelled second antibody)

A critical study of macro solid phases for use in immunoassay systems, Part III[1])

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(Received June 7/December 19, 1983)

Summary: The performance of different solid-phase luminescence immunoassays has been documented using four different assay concepts. These are CELIA (chemiluminescence immunoassay), SPALT (solid-phase antigen luminescence technique), ILMA (immunoluminometric assay) and ILSA (immunoluminometric labelled second-antibody assay). CELIA is analogous to a solid-phase radioimmunoassay and uses a labelled antigen, SPALT and ILSA use a labelled second (species-specific) antibody and ILMA a labelled substance-specific antibody, i. e. analogous to the immunoradiometric assay. Both bioluminescent and chemiluminescent labels have been used. Pyruvate kinase was used for bioluminescence and diazoluminol and N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide for chemiluminescence. Relevant quality-control parameters and reference ranges have been given for the optimised assays. Assays described are: thyroxine, thyroxine binding globulin, cortisol, caeruloplasmin, ferritin and C-reactive protein.

Luminescence immunoassays with coefficients of variation comparable with radioimmunoassay have been designed, values of under 5% being obtainable within the working range of the assay.

Beurteilung von vier verschiedenen Lumineszenz-Immunoassays:
CELIA (chemiluminescent immunoassay),
SPALT (solid-phase antigen luminescence technique),
ILMA (immunoluminometric assay) und
ILSA (immunoluminometric labelled second antibody).
Eine kritische Untersuchung von Makro-Festphasen zum Gebrauch in Immunoassay-Systemen, Teil III[1])

Zusammenfassung: Die Entwicklung und Durchführung von Lumineszenzimmunoassays werden am Beispiel von vier verschiedenen Assaykonzepten aufgezeigt. Der CELIA (Chemilumineszenzimmunoassay) verwendet markiertes Antigen, SPALT (Solid-Phase Antigen Luminescence Technique) and ILSA (Immunoluminometric Labelled Second-Antibody Assay) markierten spezies-spezifischen „zweiten" Antikörper und der ILMA (Immunoluminometric Assay) substanz-spezifischen Antikörper, die markiert sind. Sowohl Biolumineszenz (Pyruvatkinase)- als auch Chemilumineszenz (Diazoluminol und N-(4-Aminobutyl)-N-ethyl-isolu-

[1]) Part I: J. Clin. Chem. Clin. Biochem. *21*, 789–787. Part II: J. Clin. Chem. Clin. Biochem. *22*, 337–347.

minol-hemisuccinamid)-Markierungen wurden verwendet und gegenübergestellt. Qualitätskontrollkenngrößen und Referenzbereiche im Serum werden für die optimierten Assays ermittelt. Es werden Lumineszenzimmunoassays für die folgenden Substanzen beschrieben: Thyroxin, thyroxin-bindendes Globulin, Cortisol, Ferritin, Caeruloplasmin und C-reactives Protein.

Die entwickelten Lumineszenzimmunoassays zeigen dem Radioimmunoassay vergleichbare Variationskoeffizienten mit Werten unter 5% im relevanten Bereich.

Introduction

This third and final part of a study upon solid phases for immunoassay describes the assays which have been developed from the experiments described in the first two parts (1, 2). Both functional and non-functional assay systems have been presented as well as comparisons between different assay systems for the same analyte.

The assays described include those using labelled antigens and labelled first or second antibodies. The labels used are: diazoluminol and N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide, the latter being coupled via an active ester of N-hydroxysuccinamide. The synthesis of the labels has already been described (2). The assays represent different analytes (haptens or peptides) and methods. Where applicable, assays which have already been introduced into the routine laboratory are presented with relevant quality-control parameters and comparisons with the methods which they have replaced.

Materials and Methods

Materials

Antibodies

Antibodies to human liver and spleen ferritins were purchased from Atlanta (Pelfreez), Heidelberg; Proma (Seward), Augsburg; Boehringer-Ingelheim (DAKO), Ingelheim; these being the distributors in the FRG. Antibodies to human C-reactive protein and human thyroxine binding globulin were obtained from Proma and Boehringer-Ingelheim.

Antibodies to caeruloplasmin were purchased from Behringwerke, Marburg a.d.L., FRG, whereas those for cortisol and thyroxine were donations from Dr. *Mario Pazzagli*, Firenze, Italy and the Fa. Henning Berlin GmbH respectively.

Donkey anti-rabbit IgG was purchased from Wellcome Diagnostika, Burgwedel, FRG and rabbit anti-sheep IgG from Boehringer Ingelheim.

Standard materials

Ferritin standards were obtained from Travenol-Clinical Assays, Munich, FRG, C-reactive protein serum standard from Behringwerke, thyroxine and cortisol standards from Henning Berlin and Diagnostic Products Corporation (Hermann Biermann, Bad Nauheim, FRG) respectively. Human transferrin was purchased from Behringwerke, human caeruloplasmin from Serva, Heidelberg, FRG or Sigma, Munich, human thyroxine binding globulin being a gift from Prof. *K. Horn* and Dr. *R. Gärtner*, Munich.

Equipment

The luminometers used were either the LKB-1251 (LKB Instruments, Gräfelfing, FRG) or the LB-950 (Laboratorium Prof. Dr. Berthold, Wildbad, FRG). The LKB-1251 was a 25-sample semi-automatic instrument with microprocessor, the LB-950 had a 300 sample capacity and was also microprocessor controlled. Both instruments were at the time of the experiments not able to process data fully so that an off-line data-processing with a desk-top computer (CBM 8032 – Commodore Business Machines, Neu-Isenburg, FRG) and spline function programme was necessary.

Methods

CELIA – *chemiluminescent immunoassay*

This type of assay is analogous to a solid-phase radioimmunoassay in which the first antibody was coupled to a polystyrene ball (1) and in which the tracer was an antigen labelled with N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide instead of a radioactive marker. Table 1 shows the assay flow sheet for a TBG CELIA.

Tab. 1. Thyroxine binding globulin CELIA flow sheet.

50 µl sample or standard
200 µl assay buffer (0.05 mol/l Tris-HCl containing 2.50 g/l bovine serum albumin, pH 7.4
1 sheep anti human TBG coated ball
Incubate at ambient temperature for 30 min
50 µl diazoluminol labelled TBG (1:100 dilution*)
Incubate as above and wash with 2 × 1 ml 0.15 mol/l NaCl containing 0.15 ml/l Tween 20.
Transfer balls to measuring cuvettes and load luminometer. The light initiation step is identical with that shown in table 2.

* The diazotisation was analogous to the method described in l.c. (2), replacing the second antibody with thyroxine binding globulin. The dilution factor given here is the further dilution of the TBG-diazoluminol. The amount of label added to each tube was approximately 100 ng.

ILMA – *immunoluminometric assay*

The ILMA is analogous to the IRMA (immunoradiometric assay) (3) and is especially suited for assays where the antigen is either unavailable or prohibitively expensive so that a SPALT (see below) assay is out of the question. Several antibody-pairs were tested until a suitable combination was found. Table 2 shows an assay flow sheet for a ferritin ILMA.

ILSA – *immunoluminometric labelled second-antibody assay*

The ILSA is identical with the ILMA, but with one exception, and that is that a labelled second antibody is used in addition to the two substance-specific antibodies used in the ILMA. This assay was only used in cases where diazoluminol was used for labelling the antibodies, in order to achieve maximal sensitivity. Table 3 shows a flow sheet for a C-reactive protein ILSA.

Tab. 2. Ferritin ILMA flow sheet.

20 µl sample or standard
200 µl assay buffer (0.025 mol/l phosphate/0.025 mol/l Tris-HCl containing 0.5 ml/l Tween 20 and 1.25 g/l bovine serum albumin, pH 7.4
1 goat anti human liver ferritin coated ball
Incubate for 3 h on a horizontal rotator (180 min^{-1})
Wash with 2 × 5 ml 0.15 mol/l NaCl containing 0.15 ml/l Tween 20
200 µl N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide labelled rabbit anti human ferritin (1:500 dilution)*
Incubate and wash as above and transfer balls to measuring cuvettes.
Add 250 µl 0.15 mol/l NaCl and load luminometer. Initiate light reaction with 1 mol/l NaOH, 2 mg/l microperoxidase-MP 11 and 0.15 mol/l H_2O_2 (100 µl + 10 µl + 360 µl respectively). Integrate the light signal over 20 s

* This represents the dilution of the labelled antibody after coupling to N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide using the same method for labelling donkey anti rabbit IgG – see l.c. (2).

Tab. 3. C-reactive protein ILSA flow sheet.

10 µl sample or standard, 1:10 dilution in assay buffer (0.05 mol/l Tris-HCl containing 2.5 g/l bovine serum albumin, pH 7.6)
200 µl assay buffer
1 sheep anti human C-reactive protein coated ball
Incubation 60 min at 37 °C in a water bath, followed by 2 × 5 ml wash with assay buffer
200 µl rabbit anti human C-reactive protein (1:500 dilution)
Incubate and wash as above
300 µl diazoluminol labelled donkey anti rabbit serum (1:60 dilution*)
Incubate as above, wash and transfer balls to measuring cuvette. Load luminometer and proceed as in table 2.

* represents the further dilution of the labelled antibody as prepared in l.c. (2).

SPALT – *solid phase antigen luminescence technique*

The SPALT principle has been described in detail elsewhere (4, 5), and only the variations are briefly described here. The SPALT assay can be set up as either a competitive or sequential assay, depending upon whether the solid phase antigen is added together with the first antibody and sample or after a pre-incubation of first antibody and sample. Tables 4 and 5 show competitive and sequential SPALT assays for caeruloplasmin and thyroxine respectively.

Assay semi-automation

The assays were originally set up in 55 × 12 mm polystyrene tubes which increased the time needed for pipetting and wash steps as each tube had to be processed seperately. To increase throughput and reduce the workload assays were set up in either 20 or 60 well trays (cf. Abbott hepatitis kits). The wash steps were carried out using a Pentawash multiple wash device (Abbott Laboratories) where 5 wells were washed simultaneously with 5 ml wash solution. The antigen-antibody reaction was speeded up by incubating the trays on a horizontal rotator at 180 min^{-1}.

Tab. 4. Caeruloplasmin SPALT flow sheet.

50 µl sample or standard, 1:25 dilution in assay buffer (see table 2)
150 µl rabbit anti caeruloplasmin (1:500 dilution)
Incubate at ambient temperature for 10 min*
1 human caeruloplasmin coated ball
Incubate for 50 min on horizontal rotator (180 min^{-1}) at ambient temperature
Wash with 2 × 5 ml 0.15 mol/l NaCl containing 0.15 ml/l Tween 20
200 µl donkey anti rabbit IgG labelled with N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide (1:150$^+$ dilution)
Incubate for 60 min on rotator and wash as above. Transfer balls to measuring cuvettes and proceed as in table 2.

$^+$ represents dilution of labelled antibody as prepared in l.c. (2).
* this step improved the precision of the assay.

Tab. 5. Thyroxine SPALT flow sheet.

25 µl sample or standard
150 µl assay buffer (0.05 mol/l Tris-HCl containing 1.58 mmol/l 8-anilino-1-naphthalene sulphonic acid (ammonium salt), pH 7.5
100 µl rabbit anti thyroxine (1:500 dilution)
Incubate for 60 min at ambient temperature
1 Transferrin-thyroxine coated polystyrene ball*
Incubate as above, but on horizontal rotator and wash with 2 × 1 ml wash buffer (0.05 mol/l Tris-HCl containing 0.15 ml/l Tween 20, pH 7.5)
300 µl donkey anti rabbit IgG labelled with either pyruvate kinase or diazoluminol (1:50 dilution)
Incubate for 60 min on horizontal rotator and wash with 1 ml wash buffer followed by 1 ml 0.15 mol/l NaCl. Transfer balls to measuring cuvettes and proceed with the light initiation step as in table 2.

* Synthesis of the thyroxine-transferrin conjugate has been described in l.c. (2).

The improvement in precision, shortening of assay time and semi-automation of all methods more than compensated for the additional cost of buying the equipment. Moreover, the trays could be re-used after washing without the precision and accuracy of the assay being influenced.

Results

Comparison of a CELIA *and* ILMA *for thyroxine binding globulin* (TBG)

Figure 1 shows standard curves for a TBG-CELIA and a TBG-ILMA. Table 6 shows the relevant assay and quality control data. The antibody bound to the solid phase was the same in each case as was the label used, in this case diazoluminol. The TBG used for labelling was identical with that used for making up the standards in both assays.

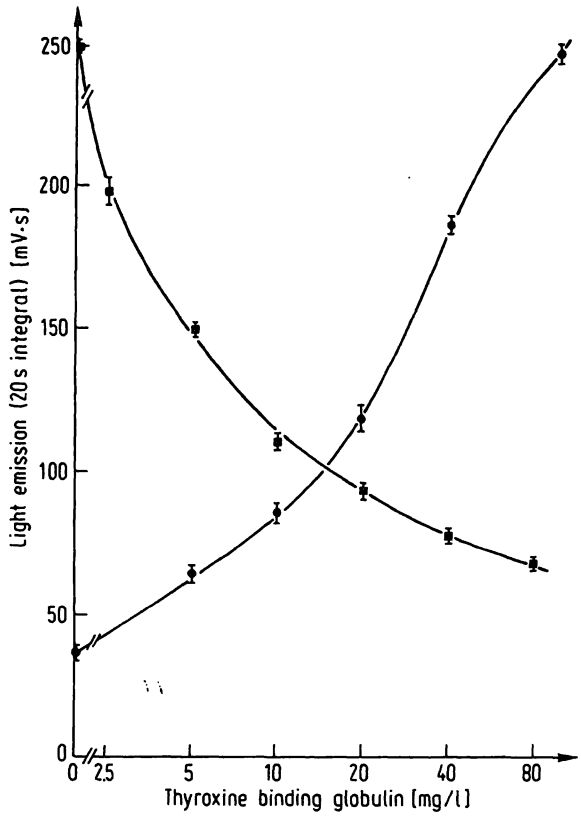

Fig. 1. Comparison between the thyroxine binding globulin CELIA (■—■) and ILMA (○—○). Both assays were measured on an LKB 1251 luminometer, the ordinate representing the integral of the light curve over 20 seconds. Each standard curve point is shown with the mean and 2 standard deviation (95% confidence-) limits.

Comparison between labelling using ILMA *and* ILSA *for ferritin as examples*

Figure 2 shows three standard curves using identical starting reagents. For the ILSA the second antibody (donkey anti-rabbit IgG) was labelled with diazoluminol (2), for the ILMAs both diazoluminol and N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide were used to label the second anti-ferritin antibody. Table 7 shows relevant data from all three curves, here with special reference to the sensitivity (lowest detectable antigen level).

Comparison of different pH values to initiate the light reaction using a serum cortisol SPALT *as an example*

Table 8 shows a comparison between three cortisol SPALT assays set up at the same time with the same reagents. The difference between the assays is that one is oxidised at pH 8.6 to initiate the light reaction, the second with sodium hydroxide, directly after addition, and the third, after the solid phase has been left in contact with sodium hydroxide for 2 h. The assay flow sheet up until the light-reaction step is shown in table 9.

Tab. 6. Comparison of thyroxine binding globulin (TBG) CELIA and ILMA using diazoluminol as label.

Parameter	Assay 1+	Assay 2
Assay type	CELIA	ILMA
Sample size (µl)	50	1 (20 µl 1:20 dilution)
Standard matrix	Buffer	Buffer
Standard curve		
Counts++		
Zero standard (B_0)	250	37
5 mg/l standard (B_5)	150	65
80 mg/l standard (B_{80})	67	245
Ratios		
B_0/B_{80}	3.73*	0.151
B_5/B_0	0.60	1.76
B_{80}/B_0	0.268	6.62*
Quality control		
Control sera (mg/l)**		
H1 (14 ± 3) (mean ± s.d.)	above 80	13.6
H2 (26 ± 5)	above 80	27.3
H3 (37 ± 6)	above 80	35.9
Correlation data***	with RIA	
No. of samples	–	89
Correlation cofficient r	–	0.985
Intercept a_{yx} (mg/l)	–	−2.74
Slope b_{yx}	–	0.976
Range of values used (mg/l)	–	10–43

\+ Both assays were performed simultaneously.
++ Expressed as mV · 20 s (integral)
* These values represent the dynamic range for the CELIA and ILMA respectively.
** The CELIA, although giving a standard curve, was unable to measure serum samples, and was therefore abandonned. Target values in brackets.
*** The correlation coefficient and related data was determined from a comparison with the Henning TBG-RIA, results from 6 assays being used. The RIA values were entered as x.

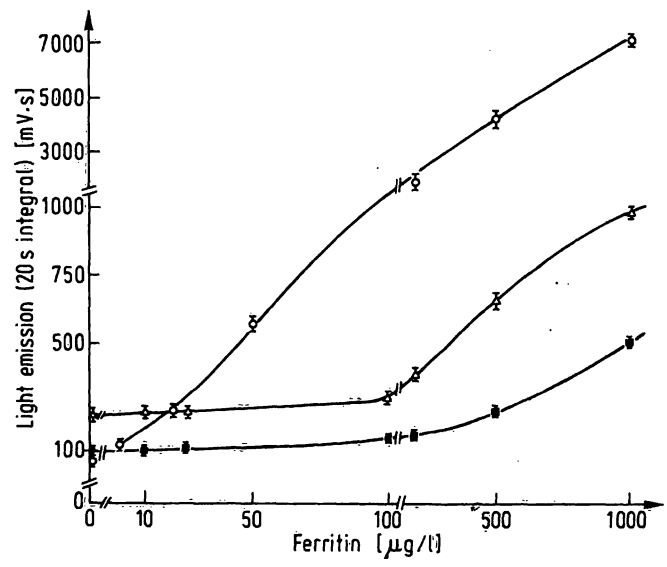

Fig. 2. Comparison of three ferritin assays using diazoluminol (△—△ ILSA; ■—■ ILMA) and N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide (○—○ ILMA) labelled antibodies. Please note that the scales are not linear. The symbols and their significance are as in figure 1.

Tab. 7. Comparison of ferritin ILMA and ILSA using diazoluminol and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide (ABEI-H) as labels – relevant assay data.

Parameter	ILMA-1	ILMA-2	ILSA
Label	Diazo-luminol	ABEI-H	Diazo-luminol
Counts+			
Zero standard (B_0)	103	78	222
5 µg/l standard (B_5)	–	125	–
10 µg/l standard (B_{10})	108	–	238
200 µg/l standard (B_{200})	155	1940	370
1000 µg/l standard (B_{1000})	507	4210	965
Ratios of counts			
B_5/B_0	–	1.60	–
B_{10}/B_0	1.05	–	1.07
B_{200}/B_0	1.50	24.9	1.67
B_{1000}/B_0++	4.92	54.0	4.35
Spread of counts+*			
$B_{1000} - B_0$	404	4132	743
Sample size (µl)	100	20	100
Assay time (h)	24	6	26
Control serum values (µg/l)**			
D4 – target value 16 ± 1.9	30	16.4	19.0
D5 – target value 53 ± 4.2	55.0	48.3	52.4
D6 – target value 180 ± 6	192	164	177

+ Integral over 20 s, given in 1000 "Berthold light units"
++ Expression of the dynamic range of the assay
* Represents the actual spread of counts between the lowest and highest standard.
** Values given as mean ± 1 standard deviation. The values given are the mean of duplicate values.

Tab. 8. Effect of pH and exposure time to sodium hydroxide on the light emission using the cortisol SPALT as an example.

Parameter	pH 8+	pH 13+ immediate	pH 13+ after 2 h
Light emission of zero standard (B_0)	3294*	5391	20444
Light emission of unspecific binding (UB)	85*	103	441
Light emission of 2650 nmol/l standard (S_{2650})	837*	1385	6726
Ratio UB/B_0	0.03	0.02	0.02
Ratio S_{2650}/B_0	0.25	0.26	0.33
50% intercept (nmol/l)	660	473	908
Control serum D4 (nmol/l)++	154	132	143
Control serum D5	622	638	539
Control serum D6	1523	1413	1721

* Results in 1000 "Berthold Light Units"
+ All assays were set up at the same time. The results in column 2 were obtained when the tubes were processed directly after NaOH addition, those in column 3 when the light initiation step took place 2 h after NaOH addition. The results in column 1 were obtained when 0.5 mol/l phosphate buffer replaced the 1 mol/l NaOH.
++ Results expressed are the mean of duplicate tubes.

Tab. 9. Cortisol SPALT flow sheet.

10 µl sample or standard
200 µl rabbit anti cortisol, 1:4000 in assay buffer (0.1 mol/l phosphate containing 0.1 mol/l sodium salicylate, pH 7.5)
1 cortisol-3-carboxymethyloxime-ovalbumin coated ball

Incubate on horizontal rotator (180 min^{-1}) for 35 min at ambient temperature
Wash with 2 × 5 ml 0.15 mol/l NaCl containing 0.15 ml/l Tween 20

200 µl N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide labelled donkey anti rabbit IgG (1:180 dilution*)

Incubate as above, but for 45 min, wash as above and proceed as described in table 2 for the ball-transfer and light-initiating steps

* See table 4.

Comparison between pyruvate kinase and diazoluminol as label for the second antibody using a thyroxine SPALT *as an example*

The assay flow scheme has already been shown in table 5. Figure 3 shows standard curves for both assays, the relevant assay data being shown in table 10.

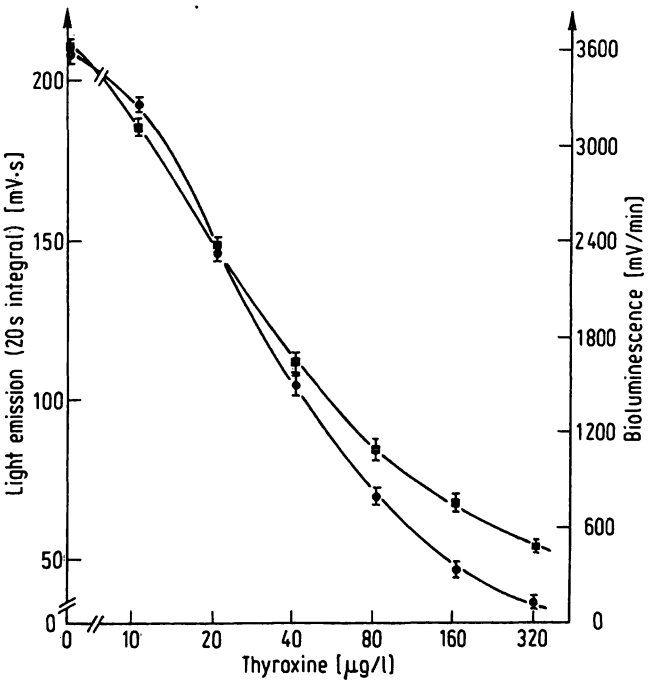

Fig. 3. Comparison of use of a bioluminescent (pyruvate kinase ■—■) label and a chemiluminescent label in a thyroxine SPALT. Both assays were measured on an LKB 1250 luminometer, the left-hand ordinate showing the chemiluminescent signal as 20 s integral, the right-hand ordinate the slope of the ATP-generation curve, expressed as mV/min.

Tab. 10. Comparison between a chemiluminescent and bioluminescent labelled SPALT assay for serum thyroxine.

Parameter	Assay 1*	Assay 2
Label	Diazoluminol	Pyruvate kinase
Standard curve		
Counts		
Zero standard (B_0)	240	3780
12 nmol/l standard (B_{12})	222	3280
410 nmol/l standard (B_{410})	66	674
Unspecific binding (UB)	31	202
Ratios		
UB/B_0	0.130	0.053
B_{12}/B_0	0.925	0.868
B_{410}/B_0	0.275	0.178
B_0/B_{410}++	3.64	5.61
Intercepts (nmol/l)		
80%	21.4	23.4
50%	64.8	57.4
20%	313	285
Quality control		
Serum A (27 ± 5) nmol/l**	21.1	24.0
Serum B (74 ± 10)	81.8	71.6
Serum C (166 ± 16)	165	157

+ Counts for Assay 1 mV · 20 s (integral), for Assay 2 mV/min (slope)
++ Expression of the assay dynamic range
* Assays set up at the same time and measured manually on an LKB 1250 Luminometer (see l.c. (1) and (2))
** Target values ± 1 standard deviation – results are the mean of duplicate determinations.

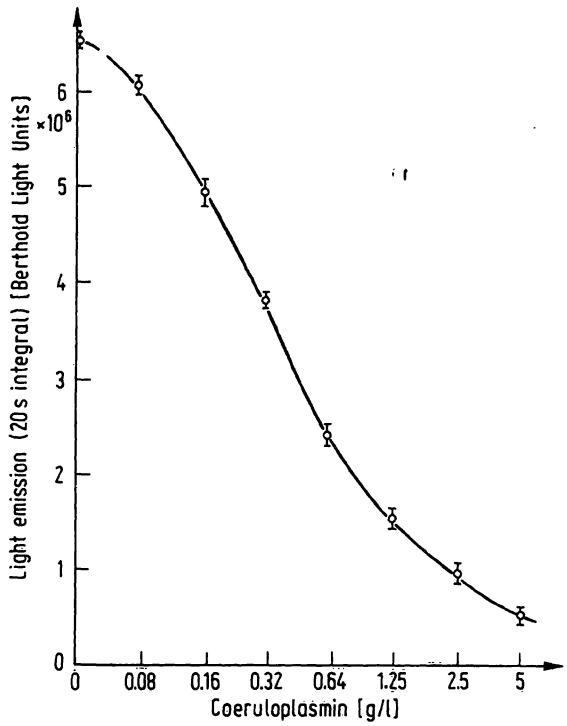

Fig. 4. Caeruloplasmin SPALT measured on the Berthold LB-950 luminometer. The ordinate represents the 20 s integral, expressed here in "Berthold light units" × 10^6, as this luminometer employs photon counting techniques rather than the current amplification method used in the LKB instruments.

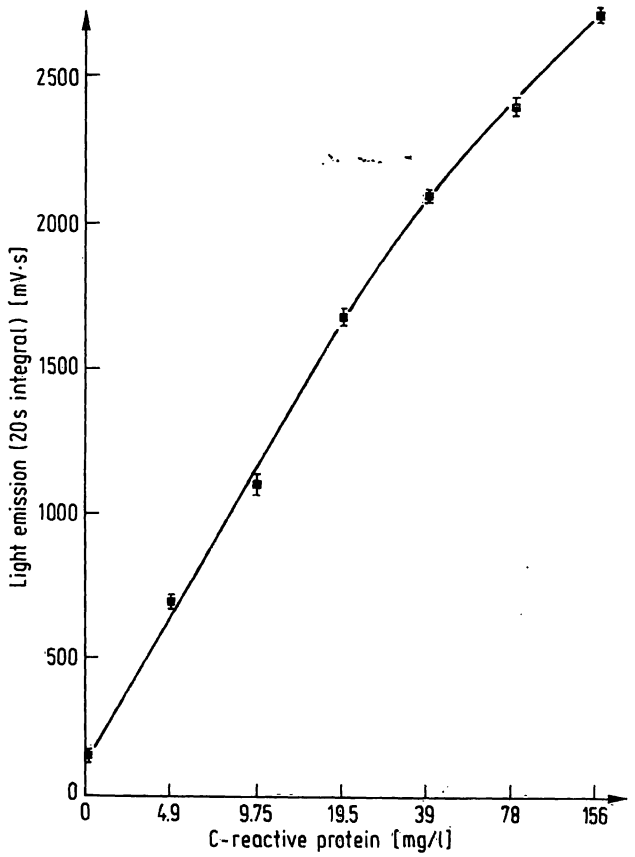

Fig. 5. C-reactive protein ILSA, using a diazoluminol labelled second antibody (donkey anti-rabbit IgG). This assay was measured on an LKB 1251 luminometer, the ordinate showing the 20 s integral value.

Clinical examples of routine luminescence immunoassays – data from a caeruloplasmin SPALT, *a C-reactive protein* ILSA *and a ferritin* ILMA

Figures 4 and 5 show typical standard curves for caeruloplasmin and C-reactive protein respectively. The label for the caeruloplasmin SPALT was N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide, for C-reactive protein, diazoluminol. In both cases, the second antibody was labelled donkey anti-rabbit IgG. Table 11 shows clinical data obtained for different patient groups. The correlation between the ferritin assay here described and the radioimmunoassay used as its predecessor in the routine determination was excellent, ($n = 91$, $r = 0.992$, $a_{yx} = 0.071$ and $b_{yx} = 0.999$) when the same standards were used in each assay. Here the radioimmunoassay values were entered as x, those from the ILMA as y. The range of the values used for this calculation were 5–570 µg/l for the RIA and 3–567 µg/l for the ILMA.

Tab. 11. Assay parameters and values from three experimental groups for caeruloplasmin, C-reactive protein and ferritin assays.

Parameter	Caerulo-plasmin	C-reactive protein	Ferritin
Assay type	SPALT	ILSA	ILMA
Label	ABEI-H⁺	Diazoluminol	ABEI-H⁺
Standard matrix	Buffer	Serum	Buffer
Standard	Human CP⁺	Human CRP⁺	Human LF⁺
Reference range (serum)	0.15–0.65 g/l	under 5 mg/l	30–300 µg/l
Assay working range⁺⁺	0.15–1.25 g/l	2–160 mg/l	5–1000 µg/l
Mean precision (%)⁺⁺⁺	5.8 (n = 205)	4.2 (n = 99)	4.3 (n = 1254)
Lower detection limit	<0.08 g/l	<1 mg/l	<2 µg/l
Experimental groups			
Crohn/Colitis			
Range	0.14–0.53 g/l	1.72–60.2 mg/l	2–911 µg/l
Median	0.30 g/l	22.9 mg/l	23.8 µg/l
No. of elevated values*	0	18	4
No. of reduced values*	1	–	23
No. of patients in group	12	21	40
*Tumour bearers***			
Range	0.13–2.34	0.8–139	3–2440
Median	0.44	11.8	152
No. of elevated values	12	14	14
No. of reduced values	1	–	4
No. of patients in group	35	18	42
Blood donors – unselected			
Range	0.06–1.01	0.5–10.7	5–240
Median	0.26	0.91	58
No. of elevated values	3	3	0
No. of reduced values	4	–	16
No. in group	45	64	84

⁺ CP = caeruloplasmin, CRP = C-reactive protein, LF = liver ferritin, ABEI-H = N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide

⁺⁺ Range in which the mean precision is under 10%

⁺⁺⁺ Actual precision within the working range – mean of all patients (n = . . .)

* Values lying below or above the established reference range.

** Pre-operative values – only patients with surgically confirmed tumours used in this analysis.

Discussion

The aim of this paper has been to put into practice some of the experiments performed in the first two parts of this series (1, 2). A comparison of both labels and methods has been made using a wide variety of antigens as ligands. Detailed clinical trials and evaluation results have been avoided, results being limited to a few assays, especially those shown in table 11.

The future of the luminescence immunoassay lies in its field of application, especially where its role as a practical alternative to routine radioimmunoassays is concerned. The assays presented here represent the whole gamut of molecular size and concentration found in in-vitro clinical chemistry and endocrinology.

The results of the comparison between a CELIA and ILMA using the same label and components (diazoluminol and immobilised sheep anti TBG) shows the relative sensitivity and working range of the two assay types, here for thyroxine binding globulin. The larger working range of the ILMA agrees with the findings of *Hunter* (6) who compared radioimmunoassays and immunoradiometric assays for sensitivity and working range. The TBG CELIA, although giving a dose response curve when the standards were dissolved in a buffer matrix, was unable to be used for measuring serum samples, where all values lay above the highest standard. The combination of the relatively large sample volume (50 µl) and contact between serum and label may be a crucial factor with the CELIA, as the TBG ILMA, where a much smaller sample (1 µl) was used, and where serum and label did not come into contact, measured correctly. Similar problems were encountered with a thyroxine CELIA using larger serum volumes (50 µl) and low-titer low affinity antibodies (*Gadow*, unpublished results).

The comparison between the different labels is well demonstrated in the case of the ferritin assays (table 7, figure 2). Although the detection limits of free diazoluminol and N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide were similar (2), the N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide label allowed an assay to be developed which was sensitive enough for routine use. This was probably due to the difference in the chemical coupling methods used, the N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide active ester being under neutral and mild reaction conditions, the diazo coupling taking place in alkaline solution over a period of several hours. The ferritin assays show the relative performance of ILMA and ILSA using the same label (here diazolu-

minol). The dynamic range of the N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide labelled ferritin ILMA is far greater than that using diazoluminol as label. This is reflected in the ratio between the number of counts in the highest standard and those in the zero standard, (see tab. 7). This ratio was 54:1 for N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide labelled anti ferritin and only 4.9:1 for diazoluminol labelled anti ferritin.

Various conditions have been described for the optimal conditions for light emission which range from initiation at pH 8–9 (7) via pH 13 (4) to initiation after standing in alkaline solution for several minutes (8) to a few hours (*Kohen*, personal communication). The cortisol SPALT (see tab. 8 and 9) was used to compare the effect of pH and alkaline incubation on the light output. Although the signal increases between pH 8 and pH 13 as well as between pH 13 when measured immediately and after 2 h standing in sodium hydroxide, the signal to noise ratio, expressed in terms of the zero standard (B_0) and unspecific binding (UB) increased only slightly.

The dynamic range of the assay, expressed as the light signal given by the zero standard and that given by the highest standard, remained almost unchanged between pH 8 and pH 13, and was noticably reduced after prolonged alkaline incubation before light initiation.

The values of the control sera lay within the expected limits for all assays.

The thyroxine SPALT was used to demonstrate the use of bioluminescent and chemiluminescent labels. The bioluminescent SPALT had a larger dynamic range when compared with the diazoluminol labelled assay, and was potentially more sensitive, as can be seen from the intercept values (tab. 10). Both assays measured correctly in terms of the control sera. Replacement of the diazoluminol labelled second antibody by one labelled with N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide gave rise to a similar increase in the dynamic range as seen in the ferritin ILMA, so that for the routine assay, N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide was chosen as label.

As stated above, the proof of the assays is in their clinical routine application and examples have been presented for ferritin, C-reactive protein and caeruloplasmin, all of which are the only routine assay for these components. The results shown are taken from projects on patients with tumours and with *Crohn*'s disease or ulcerative colitis (see tab. 11). The screening of blood donors is part of an ongoing routine procedure, especially upon new and regular donors.

The mean precision within the working range, here derived from compound precision profiles, was acceptable and comparable with commercial tests. A further advantage of these tests was their suitability for paediatric use, the largest sample volume being 20 µl for ferritin.

One thing became clear when working with immobilised antibodies, and that was the necessity for partial purification of the native antisera before coupling to the solid phase. This was effected by precipitation of the γ-globulin fraction with 200 g/l polyethylene glycol (M_r 6000) with resolution of the precipitate in 0.02 mol/l phosphate buffer, pH 8. Subsequent passage over a DEAE-cellulose column was sufficient to give a product with acceptable purity.

To conclude, it has been shown that the application of luminescence immunoassays in routine in-vitro diagnosis is now possible, although the acceptance of such assays still depends upon the commercialisation of the methods.

Acknowledgements

The authors wish to thank Frau *Jutta Jäger* for technical assistence.

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